Developmental and age-related changes in apolipoprotein B mRNA editing in mice

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Abstract Apolipoprotein B (apoB) mRNA is modified by a post-transcriptional editing reaction (C to U) changing a glutamine (CAA) to a translational stop codon (UAA) and producing apoB-48 mRNA in mammalian liver and intestine. Developmental and age-related changes in apoB mRNA editing were studied using two mouse strains with different aging processes (SAM-R/1 with a normal aging process and SAM-P/1 with an accelerated aging process). During growth of both strains, the proportion of unedited (apoB-100) mRNA decreased from 80% in the fetal liver at the 17th day of gestation to 30% in the liver of mature 2-month-old mice. Age-associated increase in the proportion of hepatic apoB-100 mRNA was observed from the age of 18 months in the SAM-R/1 strain. In the SAM-P/1 strain, apoB-100 mRNA in the liver continued to increase from the age of 10 months to death. The profiles of developmental and age-related changes in the proportion of two serum apoB isoproteins (apoB-100 and apoB-48) followed the extent of hepatic apoB mRNA editing. Age-related changes in the extent of apoB mRNA editing in the small intestine were not observed in either strain. A slight expression of apoB was detected by reverse transcriptase-polymerase chain reaction in the kidney, stomach, and colon, and age-associated change in the extent of editing was observed in the kidney. These correlated changes in apoB mRNA editing and serum apoB proteins suggest that RNA editing may be one mechanism involved in the regulation of lipoprotein biogenesis in biological development and in senescent mice. An age-associated decrease in the extent of hepatic apoB mRNA editing and increases of the proportion of serum apoB-100 protein were observed in senescent mice.—**Higuchi, K., K. Kitagawa, K. Kogishi, and T. Takeda.** Developmental and age-related changes in apolipoprotein B mRNA editing in mice. *J. Lipid Res.* 1992. **33:** 1753–1764.

Supplementary key words serum apoB-100 and apoB-48 • aging and development • Senescence Accelerated Mouse (SAM)

Apolipoprotein B (apoB) plays a key role in the assembly, secretion, and metabolism of plasma lipoproteins and in the regulation of circulating lipids as well as in processes related to atherosclerosis (1, 2). The two major forms of apoB protein with different molecular weights are apoB-100 and apoB-48 in humans (3), rats (4), and mice (5). A high molecular weight form, apoB-100, which is a ligand for the low density lipoprotein (LDL) receptor, is produced mainly in the liver of humans and rats. The low molecular weight form, apoB-48, is produced mainly in the small intestine in humans and in the intestine and the liver in rats (6, 7).

Both forms of apoB are encoded by a single gene (8). A 14.1-kilobase (kb) apoB mRNA is modified by a post-transcriptional editing reaction in which a single base change from cytosine (C) to uracil (U) in apoB-100 mRNA converts CAA codon coding for glutamine at residues 2153 to a premature in-frame translational stop codon (UAA) (9–12). This RNA editing producing a apoB-48 mRNA in the human intestine and in the rat liver and intestine is one mechanism involved in the control of lipogenesis (13, 14). Recent studies showed that the administration of thyroid hormone, fasting, and the development of newborn rats modified the synthesis of two species of apoB proteins, in association with changes in the extent of apoB mRNA editing in the liver (13, 15–17).

We examined changes in the extent of apoB mRNA editing in the mouse from the 17th day of gestation to 26 months after birth and compared findings with changes in serum apoB proteins, using two inbred strains of mice (SAM-R/1 and SAM-P/1) with different aging processes. Senescence Accelerated Mouse (SAM) is a group of inbred mouse strains developed by Takeda and co-workers (18) as a murine model of accelerated senescence. The accelerated senescence-resistant mice (SAM-R/1 strain) have a normal aging process. The accelerated senescence-prone mice (SAM-P/1 strain) have a shorter life span and an earlier onset and irreversible advancement of senescence, as revealed by analysis of aging dynamics such as

Abbreviations: ANOVA, one-way analysis of variance; apoB, apolipoprotein B; RT-PCR, reverse transcriptase-polymerase chain reaction; LDL, low density lipoproteins; bp, base pair; kb, kilobase; PVDF, polyvinylidene difluoride; ABC, avidin biotinylated horseradish peroxidase complex.

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Gompertz function, survival curves, and grading score system (19, 20).

We found an age-associated decrease in the extent of apoB mRNA editing and increases of the proportion of apoB-100 mRNA in the liver and apoB-100 protein in serum of the senescent mice. These observations provide insight into aspects of lipogenesis in senescent animals and into the pathogenesis of cardiovascular disease.

MATERIALS AND METHODS

Animals

The SAM-R/1 strain with a normal aging process and the SAM-P/1 strain with an accelerated aging process were developed and maintained in our laboratory by sister-brother breeding, from 1968 to the present. These mice were raised under conventional conditions at 24 ± 2°C with a light-controlled regimen (12-h light/dark cycle). The survival curves for the SAM-R/1 and SAM-P/1 strains housed in our laboratory are illustrated in **Fig. 1.** The mean and maximum life spans were 18.7 and 39 months in the SAM-R/1 strain, respectively, and 8.6 and 17 months in the SAM-P/1 strain, respectively. Timed-pregnant females, neonatal and adult male SAM-R/1 and SAM-P/1 mice were fed a commercial chow containing 4% fat (CE-2, Nihon CLEA, Tokyo, Japan) ad libitum, until they were killed at a fixed time from 10 AM to 12 noon.

RNA extraction and analysis of mRNA abundance

SAM-R/1 and SAM-P/1 mice were killed at 17 and 19 days of gestation. Fetal livers and small intestines were excised and total RNA was extracted into 5.5 M guanidine-thiocyanate and purified by ultracentrifugation through a 5.7 M CsCl cushion (21). Total RNA was obtained from the liver and small intestine of the dams and from fetal membrane and placenta. Liver and intestinal RNA was isolated from neonatal male mice 1, 4, 7, 10, 15, 20, 25, 30 days after birth and adult male mice 2, 5, 8, 10, 12, 14, 18, 22, 26 months of age. RNA was also isolated from various tissues in young (5-month-old) and old (14-month-old) male SAM-P/1 mice. RNA was suspended in 70% ethanol and stored at −80°C. ApoB mRNA abundance was determined by quantitative slot-blot analysis. Three different amounts of total RNA (0.75, 1.50, and 3.00 μg) of 15 samples and one internal control (pooled liver RNA isolated from 2-month-old SAM-R/1 male mice) were applied on each nitrocellulose membrane. After fixation by ultraviolet crosslinker (Stratagene, La Jolla, CA), membranes were prehybridized for 4 h and then hybridized overnight at 42°C using 50% (vol/vol) deionized formamide, 5 × SSC (where 1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 2 × Denhardt's solution, 50 μg/ml salmon sperm DNA, and 0.1% SDS. The mouse apoB cDNA clone encoding the middle region of mouse apoB mRNA was a 1.8 kb long EcoRI fragment isolated from a liver cDNA library of C57BL/6J mice in phage λgt11 (Clontech Laboratories Inc., Palo Alto, CA). The apoB cDNA probe was labeled with ^{32}P by random priming and was used at 2 × 10^6 cpm/ml in the hybridization reactions. Washing included a 3 × 5-min wash at room temperature in 2 × SSC, 0.1% SDS, a 30-min wash at 65°C in 2 × SSC, 0.1% SDS, and a final 1-h wash at 65°C in 1 × SSC, 0.1% SDS. Membranes were exposed to Fuji XR films at −80°C. Autoradiographs were analyzed by video densitometric image analyzer Luzex 3U (Nikon, Tokyo, Japan). The regression coefficients of linear plots of samples were used to calculate the apoB mRNA levels. All membranes were re-probed with β-tubulin cDNA (22) to normalize quantitative and qualitative variations in RNA samples. Northern blot analysis was done using

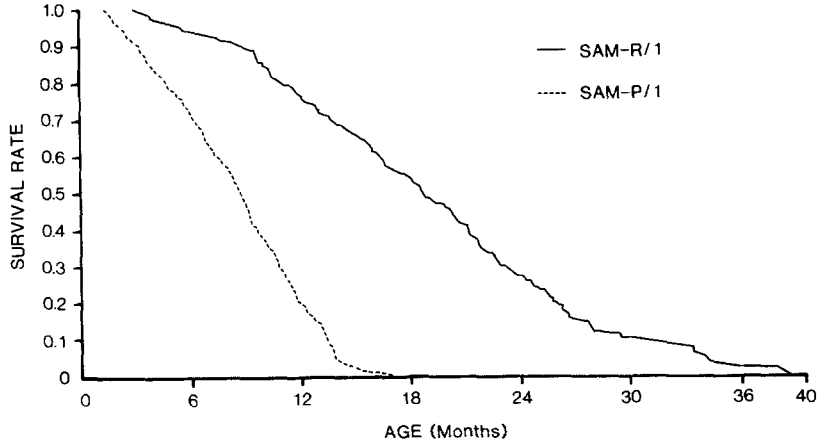

Fig. 1. Survival curves for male and female SAM-R/1 strain (solid line, n = 993) and the SAM-P/1 strain (dashed line, n = 397). Mice were maintained under conventional and controlled environmental conditions.

1.0% agarose gels containing 6.0% (vol/vol) formaldehyde (23).

Analysis of apoB mRNA editing

The extent of apoB mRNA editing was determined using the complementary techniques of *a)* differential hybridization and *b)* primer extension of reverse transcribed RNA (24) after amplification by the polymerase chain reaction (RT-PCR).

Total cellular RNA was subjected to DNase I (RNase-free, Takara Biochemical Inc. Kyoto, Japan) to remove any genomic DNA. The first strand cDNA was synthesized from 5 μg of total RNA using random hexamer priming and 14 units of AMV reverse transcriptase (Pharmacia, Uppsala, Sweden). PCR amplification was then done using one-tenth of cDNA and two mouse apoB-specific primers (SO-mB1 and SO-mB2). SO-mB1: 5' TCTGAATTCATCTGACTGGGAGAGACAAGTAG 3', 32-mer is the coding strand, annealing 132–164 nucleotides upstream from edited cytosine (nucleotide 6666) of the apoB mRNA; SO-mB2: 5' GTTGATCATAATTTC-TTTAATATACTGATCAA 3', 32-mer is complementary to apoB mRNA and locates 5–37 nucleotides downstream from nucleotide 6666. The mixture in a final volume of 100 μl, with conditions as specified in the GeneAmp kit (Perkin-Elmer/Cetus, Norwalk, CT) was incubated at 94°C for 6 min, followed by 40 cycles of successive denaturation (94°C for 1 min), annealing (55°C, 2 min), and extension (72°C, 2.5 min), followed by a final 10-min extension at 72°C (25). The 201 base pair (bp)-long PCR products were incubated with RNase A, extracted with phenol–chloroform, precipitated with ethanol, and dissolved in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, pH 8.0 (TE).

a) PCR products (5 ng/slot) were denatured in 0.5 N NaOH, 1.5 M NaCl, and neutralized in 0.5 M Tris-HCl, pH 8.0, 1.5 M NaCl and applied onto a nylon membrane (Gene Screen Plus, NEN Products, Boston, MA), using a slot-blot apparatus (Bethesda Research Laboratories, Bethesda, MD), then hybridized with an apoB cDNA probe and two oligonucleotide probes (SO-mBGln and SO-mBStop). SO-mBGln: 5' GATCAAATTGTATCGCGTAT 3', 20-mer completely complementary to unedited mouse apoB (apoB-100) mRNA, and SO-mBStop: 5' GATC-AAATTATATCGCGTAT 3', 20-mer complementary to edited apoB (apoB-48) mRNA were ^{32}P-labeled with T4 polynucleotides kinase. After 4 h prehybridization in 5 × SSC, 2 × Denhardt's solution, 0.125 mg/ml salmon sperm DNA, 0.125 mg/ml yeast tRNA, and 0.1% SDS at 45°C, the hybridization reaction was allowed to proceed at 45°C for 16 h in hybridization solution with 2 × 10^6 cpm/ml of labeled oligonucleotide probe and 50 μg/ml yeast tRNA. A 3 × 5-min wash at room temperature in 5 × SSC, then a 30-min wash in 5 × SSC at 54.5°C (SO-mBGln) or 51.0°C (SO-mBStop) was done. Filters were exposed to KODAK XAR film at −80°C and integrated density of each slot was determined using a video densitometer. The extent of apoB mRNA editing was calculated using control PCR products amplified from plasmid DNAs containing either CAA or TAA at codon 2153.

b) PCR products of 255 bp were amplified from cDNA using primers, SO-mB1 and SO-mB3 (5' TTAAGC-TTTTCAATGATTTCATCAATAATATT 3' 32-mer annealing 59–91 nucleotides downstream from nucleotide 6666) and used for primer extension analysis. Approximately 2.0 ng of amplified DNA was denatured at 95°C, annealed to 200 pg of ^{32}P-labeled anti-sense mouse apoB oligonucleotide primer SO-mB4 (5' ATCATAATTATC-TTTAATATACTGA 3' 25-mer, 5' end at 32 nucleotides downstream from nucleotide 6666) at 37°C for 1 h in 50 mM PIPES, pH 6.4, 0.2 M NaCl. After ethanol precipitation, the samples were suspended in 50 mM Tris-HCl, pH 8.4, 8 mM $MgCl_2$, 30 mM KCl, 10 mM dithiothreitol, 500 μM each dATP, dCTP, dTTP, and 1.0 mM dideoxy GTP. Extension was conducted for 90 min at 42°C using 400 units of M-MLV reverse transcriptase (Bethesda Research Laboratories, Bethesda, MD). Products were analyzed by 8.0% polyacrylamide-urea electrophoresis and subjected to autoradiography at −80°C using Fuji XR films. Autoradiographs were analyzed by a video densitometric image analyzer.

Serum apoB isoprotein distribution

The levels of two molecular weight species of apoB protein (apoB-100 and apoB-48) in serum were determined using an immunoblotting method. Two μl of serum was applied to 5.0% SDS-polyacrylamide slab gel and electrophoresis was carried out at 30 mA for 5 h (5). After electrophoresis, samples were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore, Bedford, MA) at 150 mA for 24 h. Two apoB species were detected after incubation of the membrane with monospecific rabbit anti-mouse apoB antisera (diluted 1:3,000) by the avidin biotinylated horseradish peroxidase complex (ABC) method, using 3–3' diaminobenzidine tetrahydrochloride as a substrate. Antisera against mouse apoB proteins purified from delipidated mouse LDL through a Sepharose CL-6B column were prepared in a rabbit (26). The amounts of two apoB species were determined by comparing the intensity of bands corresponding to apoB-100 and apoB-48 with the bands of internal control, purified mouse apoB proteins using a video densitometer.

Statistical analysis

All data are presented as the mean ± SD. A Statistical Analysis System (SAS) package was used to analyze the data. The effects of age on apoB mRNA editing, apoB mRNA levels, and serum apoB proteins were analyzed by one-way analysis of variance (ANOVA). Comparisons be-

tween the data at each age were made using Tukey's test. In some cases Student's *t* test was used to determine the statistical difference between mean values.

RESULTS

Quantitation of the extent of apoB mRNA editing

Known proportions of apoB-100 and apoB-48 PCR products amplified from plasmid DNAs containing CAA or TAA at codon 2153, were diluted in 100-fold excess salmon sperm DNA and used as standards to test the accuracy and linearity of the two techniques used. In the case of differential hybridization, PCR products were first probed with apoB-48 specific oligonucleotide and reprobed with apoB-100 specific oligonucleotide and finally hybridized with the apoB cDNA probe to normalize variation in the amounts of DNA applied on the filter. A linear relationship was obtained between the percentages of input apoB-100 PCR products and those calculated (**Fig. 2A**). However, relatively large variations from the theoretical values were observed when proportions below 10% and those over 90% of apoB-100 PCR products were present in the standard mixture. In the case of primer extension, analysis in the presence of a high concentration of dideoxy GTP, a better linearity was obtained and fewer variations were observed, compared to findings with differential hybridization (Fig. 2B). A greater accuracy was observed in the range below 10% of apoB-100 PCR products. Considering the quantitative characteristics of the two methods, we determined the percentage of apoB-100 PCR product in each sample, using both methods, in duplicate, and took an average of four values when the percentage of apoB-100 PCR products was between 10% and 90%. When the percentage of apoB-100 PCR products was below 10% or over 90%, we used only primer extension techniques, in triplicate.

Developmental and age-related changes in apoB mRNA editing in the liver and small intestine

Hepatic and intestinal RNA was isolated from the fetal and neonatal SAM-R/1 mice with a normal aging process and the extent of RNA editing was determined (**Fig. 3**). In the liver, the proportion of unedited apoB-100 mRNA to the total apoB mRNA was 79.0% on day 17 of gestation (4 days before birth), then the proportion decreased gradually with progress in gestation and postnatal growth. The proportion of apoB-100 mRNA decreased to 30.0% on day 30 and 26.3% at 2 months after birth. The proportion of apoB-100 mRNA maintained a constant value until age 14 months; however, it did increase significantly in an age-associated manner in mice over 18 months of age ($P < 0.01$, ANOVA). The values at 18, 22, and 26 months were significantly higher than values at 12 months of age ($P < 0.05$, Tukey's test).

In the intestine, 45.8% of total apoB mRNA was apoB-100 mRNA on day 17 of gestation and the proportion decreased to 12.6% on the first postnatal day, a value close to that in adult mice. The proportion of apoB-100 mRNA did not change with growth, maturation, and senescence.

In the SAM-P/1 mice with an accelerated aging pro-

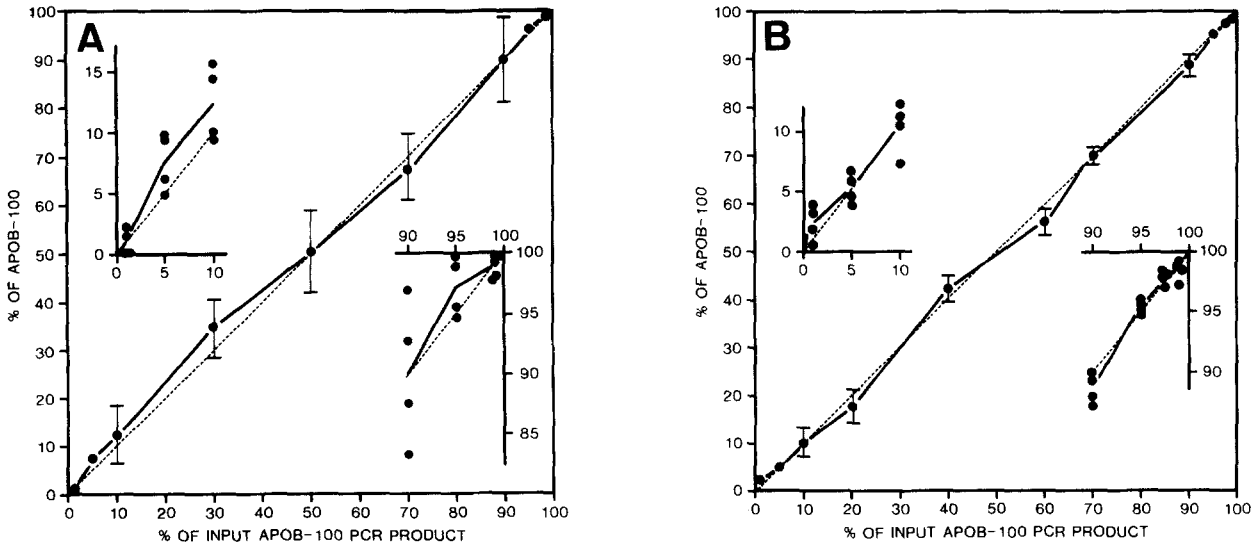

Fig. 2. Quantitation of apoB-100/total apoB PCR products by (A) differential hybridization method and (B) primer extension method. The points and bars represent the mean ± 2 SD. The dotted diagonal represents the theoretical line. Insets present data at the extreme upper and lower ends of curves, in an expanded scale.

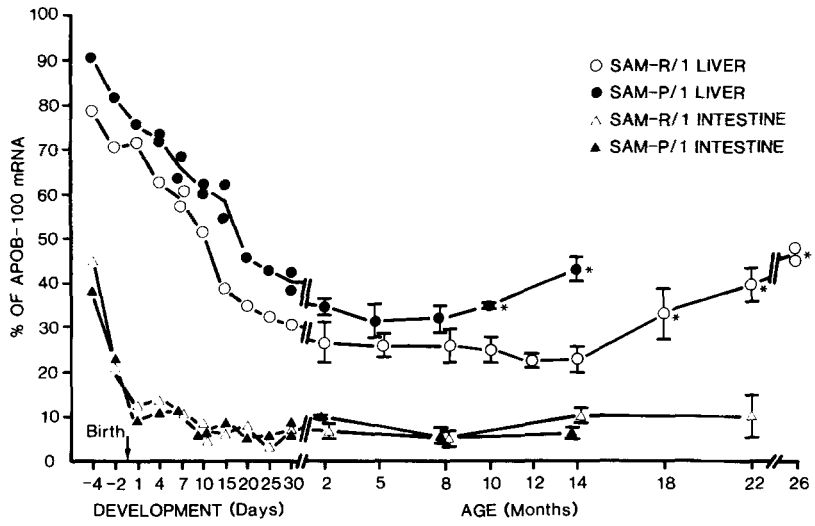

Fig. 3. Developmental and age-related changes in apoB mRNA editing in mice. Proportion of apoB-100 (unedited) mRNA to total apoB mRNA in the liver and small intestine of the male mouse during the fetal, neonatal, mature, and senescent periods were determined by RT-PCR followed by differential hybridization and primer extension analysis. Each circle and triangle represents the one pooled tissue harvested from several mice during the prenatal period, one mouse tissue during neonatal periods, and the mean value of four mice during mature and senescent periods. Bars represent SD. Significant age-associated increase in the proportion of apoB-100 mRNA is shown by ANOVA for livers from both strains, during the senescent period ($P < 0.01$). Asterisks indicate significantly higher values compared with values at the age of 12 months in the SAM-R/1 strains and at the age of 5 months in the SAM-P/1 strains (Tukey's test, $P < 0.05$).

cess, developmental changes in apoB mRNA showed the same pattern seen in SAM-R/1 mice, except that values of the proportion of apoB-100 mRNA were higher throughout the period of development (from 4 days before birth to 2 months after birth). The proportion of apoB-100 mRNA was lowest in the 5-month-old mice (30.1%). After 10 months of age, the proportion of apoB-100 mRNA increased significantly ($P < 0.01$ ANOVA) and values at the age of 10 and 14 months were significantly higher than the value at age 5 months ($P < 0.05$, Tukey's test).

The extent of apoB mRNA editing in the RNA preparations isolated from placenta, fetal membrane, liver, and intestine from 3-month-old mice on the 19th day of gestation were determined. Unedited (apoB-100) mRNA constituted 98% of the apoB mRNA in placenta and fetal membrane. The proportions of apoB-100 mRNA (%) in livers of the dams were 34.82 ± 5.16 (n = 4) in the SAM-R/1 mice and 42.20 ± 6.00 (n = 4) in the SAM-P/1 mice, and these values were significantly higher than the values in age-matched females of both strains (25.78 ± 3.84, n = 4 in SAM-R/1, 31.64 ± 3.56, n = 4 in SAM-P/1, $P < 0.05$ Student's t test). Sex difference in the proportion of apoB-100 mRNA (%) in the liver was not observed between the 3-month-old female and male SAM-R/1 and SAM-P/1 mice (26.31 ± 0.80, n = 4 in male SAM-R/1, 34.58 ± 2.92, n = 4 in male SAM-P/1).

Developmental and age-related changes in apoB mRNA expression in the liver and small intestine

Quantitative slot-blots of total liver and intestinal RNA isolated from fetal, neonatal, mature, and senescent mice were examined with ^{32}P-labeled apoB cDNA and β-tubulin cDNA for normalization. **Fig. 4A** shows changes in hepatic apoB mRNA levels in the SAM-R/1 and the SAM-P/1 strains. During the developmental period until day 30, the highest levels were found on days 7 and 20 both in the SAM-R/1 and SAM-P/1 strains. After weaning (about day 25 after birth), levels increased again and reached values in adults at age 2 months, followed by constant levels during the remaining life of both strains. The changes in intestinal apoB mRNA levels, as shown in Fig. 4B, reached peaks on day 10 in SAM-R/1 and on day 7 in the SAM-P/1. The level of apoB mRNA reached values in mice aged 2 months followed by a decrease during the remaining life span, in both strains.

Relative β-tubulin mRNA levels in the liver and intestine decreased from days −4 to 1 by about 50%, were unchanged from days 4 to 10, and decreased again and reached adult levels seen in both strains at age 1 month. There was little variation in β-tubulin mRNA levels during maturation and senescence.

Northern blots of hepatic and intestinal RNA isolated

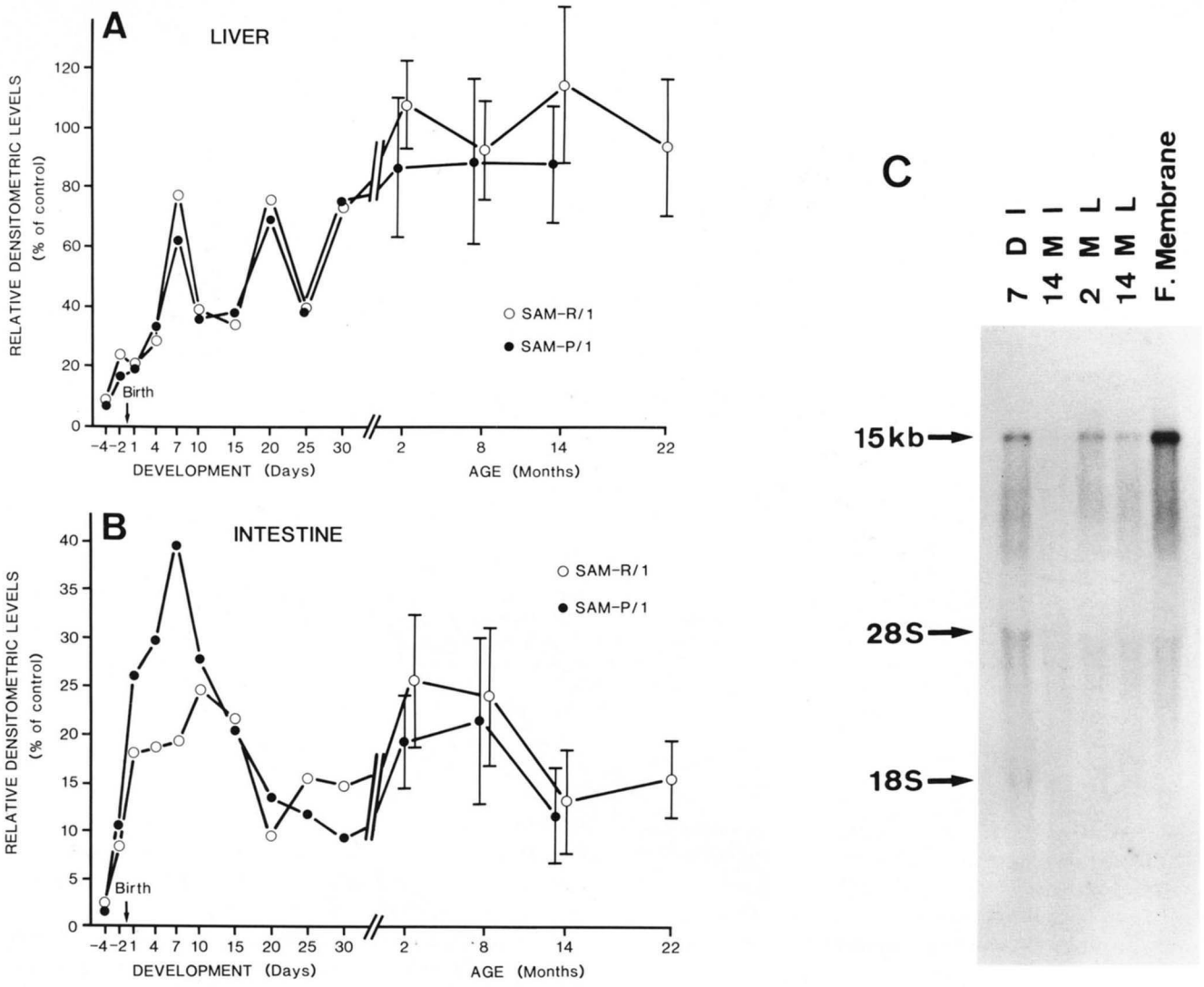

Fig. 4. Developmental and age-related changes in apoB mRNA levels in the mouse liver (A) and intestine (B). Total cellular RNA was isolated from pooled tissues harvested from several fetuses, two identically aged neonatal mice, and four mature and senescent mice. Serially diluted RNA was applied to nitrocellulose membranes for slot blot analysis. Membranes were first hybridized with the apoB cDNA probe, then rehybridized with the β-tubulin cDNA probe. Relative amounts of apoB mRNA were estimated from the slope of the signals generated by hybridization and are presented by percentage to the internal control sample (pooled hepatic total RNA isolated from the 2-month-old SAM-R/1 mice) after normalization by the amount of β-tubulin mRNA. Circles and bars indicate mean ± SD. (C) Northern blot hybridization analysis of total RNA isolated from intestines from 7-day-old and 14-month-old SAM-P/1 mice, liver from 2-month-old and 14-month-old SAM-P/1 mice, and fetal membrane in SAM-P/1 mouse at 19 days of gestation. Ten μg of total RNA was electrophoresed through 1.0% agarose, 6.0% formaldehyde gel. Migration of 15 kb apoB-100 mRNA, 28 S and 18 S ribosomal RNA is shown on the left. RNA was transferred to nitrocellulose and hybridized with ^{32}P-labeled mouse apoB cDNA coding the middle region.

from the newborn (7 days after birth), young (2-month-old), and old (14-month-old) SAM-P/1 mice and RNA isolated from fetal membrane of the SAM-P/1 mice were hybridized with an apoB cDNA probe coding the middle region of apoB (Fig. 4C). Only one major mRNA of similar apparent size (15 kb) was observed in all tissues, regardless of age of mouse or the extent of mRNA editing and abundance of apoB mRNA.

Developmental and age-related changes in serum apoB isoproteins

The levels of serum apoB isoproteins determined in immunoblot analysis are illustrated in **Fig. 5.** As shown in Fig. 5A, there was a linear relationship between the amounts of applied apoB proteins and integrated densitometric units of apoB-100 plus apoB-48 bands in the range

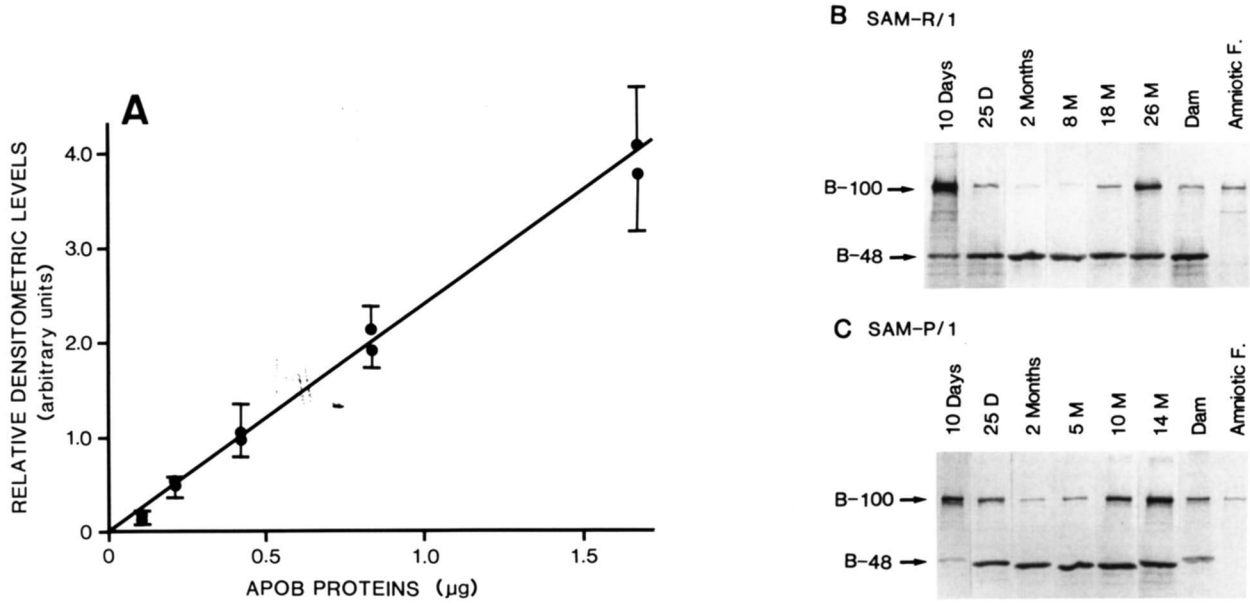

Fig. 5. Western-immunoblot analysis of apoB isoproteins in mouse serum. Two molecular weight species of apoB protein were resolved by 5.0% polyacrylamide gel electrophoresis, transferred to PVDF membranes, and incubated with monospecific rabbit antiserum against mouse apoB protein followed by detection using the ABC method. Panel A shows determination of the linearity of the immunoblot analysis of apoB proteins. Relative densitometric levels were apoB-100 plus apoB-48 bands. The points and bars represent the means ± 2 SD. Panels B and C show typical immunoblots for the SAM-R/1 (B) and SAM-P/1 (C) mouse serum at various periods and dam's serum and amniotic fluid at the 19th day of gestation. The positions of migration of apoB-100 and apoB-48 (corresponding to M_r = 550,000 and 210,000, respectively) are indicated.

from 0.1 through 1.6 μg apoB proteins. Serum concentration (mg/dl) of total apoB (apoB-100 plus apoB-48) decreased during development in both strains from 35.91 (SAM-R/1) and 30.82 (SAM-P/1) at day 10 to 15.55 (SAM-R/1) and 13.61 (SAM-P/1) at the age of 2 months (**Fig. 6A**). After age 2 months, significant age-related changes were not observed in total apoB levels (ANOVA). Serum levels of apoB-100 protein decreased to values in mice aged 2 months in both strains. Age-related increases in the serum concentration of apoB-100 were evident in both strains, by ANOVA ($P < 0.05$). The apoB-100 concentrations were significantly higher at the age of 18 and 26 months in SAM-R/1 mice and 10 and 14 months in SAM-P/1 mice, compared to the value at the age of 5 months, of each strain ($P < 0.05$, Tukey's test). The levels of apoB-48 in the serum remained unchanged during senescence. Developmental and age-related changes in the proportion of apoB-100 in total apoB protein are shown in Fig. 6B. In sera from suckling mice (day 10), apoB-100 protein was predominant (70% of total apoB) in both strains. Associated with growth or maturation, the proportions of apoB-100 protein decreased and about 20% of apoB protein in the sera was apoB-100 in both 2-month-old SAM-R/1 and SAM-P/1 strains. In sera from SAM-R/1, the low level of apoB-100 remained up to age 14 months, then the proportion of apoB-100 protein began to increase from age 18 months and reached 46.6% in the very old mice (26 months). On the other hand, in sera from SAM-P/1, the lowest proportion of apoB-100 protein was observed at 2–5 months of age to be followed by a progressive increase in the proportion of apoB-100 protein and reaching 54.4% in the 14-month-old mice. These developmental and age-related changes in apoB isoproteins followed a pattern similar to that observed in apoB mRNA editing in the liver (compare with Fig. 3).

In the apoB species in the amniotic fluid at the final stage of gestation, only apoB-100 protein was detected in both strains of mice (2.35 mg/dl, n = 2 in SAM-R/1 and 2.33 mg/dl, n = 2 in SAM-P/1) (Fig. 5B). In the sera from 3-month-old dams at this stage, both apoB species were present, and the proportion of apoB-100 protein (%) was higher in dams (22.51, n = 2 in SAM-R/1 and 43.03, n = 2 in SAM-P/1) than in the age-matched nonpregnant mice (14.64, n = 2 in SAM-R/1 and 34.88, n = 2 in SAM-P/1). Serum concentrations of apoB proteins did not differ (15.40 mg/dl and 16.75 mg/dl in the SAM-R/1 and SAM-P/1 dams, respectively, and 16.80 mg/dl and 14.85 mg/dl in the nonpregnant female SAM-R/1 and SAM-P/1 mice, respectively).

ApoB mRNA editing in tissues with small expression of apoB mRNA

Slot-blot analysis using 15 μg of total RNA isolated from brain, colon, heart, kidney, lung, muscle, pancreas,

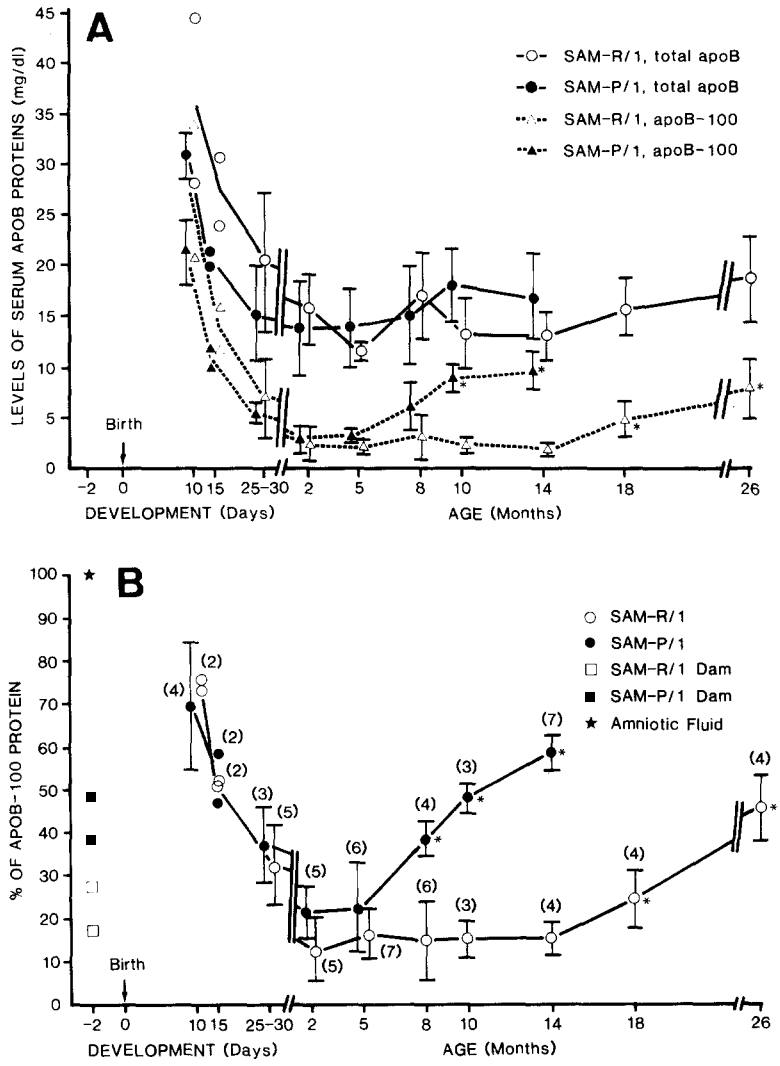

Fig. 6. Developmental and age-related changes in the distribution of apoB isoproteins on mouse serum. (A) Serum concentration of total apoB (apoB-100 + apoB-48) and apoB-100 protein. (B) Percentage of apoB-100 protein to total apoB proteins. Points and bars represent means ± SD and the figures in parentheses in panel B indicate the number of mice of each age. Significant age-associated increase in the concentration of apoB-100 (panel A) and percentage of apoB-100 protein (panel B) was noted by ANOVA ($P < 0.01$) in both strains. Asterisks indicate a significantly higher value compared with the value at the age of 5 months in the SAM-R/1 and SAM-P/1 strains (Tukey's test, $P < 0.05$).

spleen, stomach, and testis in the young (5-month-old) and old (14-month-old) male SAM-P/1 mice showed no detectable hybridization band, using the apoB cDNA probe (data not shown). To demonstrate the expression of apoB transcripts in tissue where the endogenously transcribed copy number is low, total RNA was reverse-transcribed and a 252-bp fragment of apoB cDNA flanking the edited base (nucleotide 6666) was amplified by PCR. ApoB cDNA was amplified from RNA isolated from kidney, stomach, and colon. On the other hand, no detectable DNA fragment was amplified from RNA isolated from other tissues and control reactions (without prior reverse transcriptase). The extent of apoB mRNA editing in these tissues was then examined by primer extension analysis, using RT-PCR products (**Fig. 7**). ApoB cDNA was amplified from the kidney of five young and old SAM-P/1 mice. The proportion of apoB-100 mRNA (%) was 47.85 ± 6.82 (mean ± SD) in young and 63.80 ± 7.70 in old mice (**Table 1**). In the stomach, the proportion of apoB mRNA was 19.32 ± 5.86 in young and 13.67 ± 6.63 in old mice. In the colon, the proportion of apoB-100 mRNA was 29.85 ± 7.16 in young and 29.36 ± 3.72 in old mice. The difference between the extent of apoB mRNA editing in young and old mice was

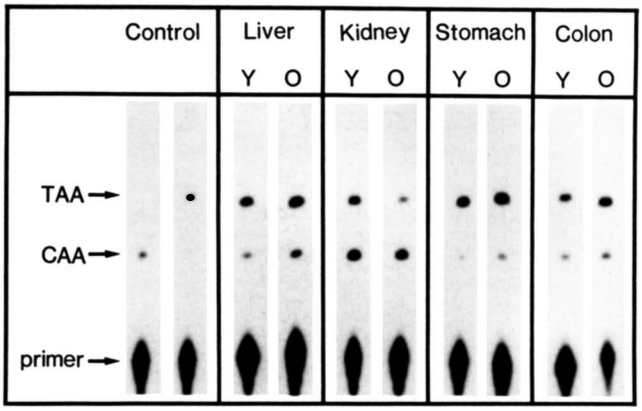

Fig. 7. ApoB mRNA editing in tissues other than liver and small intestine. The products of RT-PCR amplification of 5 μg of total RNA isolated from the kidney, stomach, colon, and liver in young (Y; 5-month-old) and old (O; 14-month-old) SAM-P/1 mice were analyzed by primer extension using a ^{32}P-labeled 25-mer oligonucleotide (SO-mB4) complementary to apoB mRNA. Control represents the primer extension analysis for PCR products amplified from two plasmid DNAs containing apoB cDNA sequences with CAA or TAA at codon 2153.

statistically significant for the kidney and liver and not significant for the stomach and colon (Student's t test, $P > 0.05$).

DISCUSSION

Human plasma cholesterol concentration increases linearly with age and is accompanied by a corresponding increase in the risk of atherogenesis (27, 28). Studies on the metabolic aspects of lipids or cholesterol in senescent experimental animals have to be done in order to elucidate the pathogenesis of atherosclerosis and other age-correlated lipid disorders. ApoB-100, which contains an LDL receptor binding domain(s), and apoB-48, which is devoid of it, are physiologically different proteins and as a result, changes in the extent of apoB mRNA editing and in the proportion of two apoB isoproteins in serum may be of physiological significance in lipids and cholesterol homeostasis. We determined the apoB mRNA editing activity in major apoB-producing tissues, liver, and small intestine throughout different stages of life, using two strains of mice with different aging processes. We found an age-associated increase in unedited apoB-100 mRNA in the liver. This increase may be related to the increase in apoB-100 protein in sera of senescent mice.

In humans, primates, and rabbits, apoB mRNA editing is performed in a tissue-specific manner. Namely, unedited apoB-100 mRNA is a major mRNA species in the adult liver and apoB-100 protein is synthesized predominantly in the liver. On the other hand, edited apoB-48 mRNA is a major mRNA species in the intestine (6, 9–12). In these species, LDL in which apoB-100 protein is a major protein component is the major serum lipoprotein and atherosclerosis occurs spontaneously and can be induced by a high fat diet. In rodents, both unedited and edited apoB mRNA are present in the adult liver (29). Rats and mice have a low concentration of LDL, and high density lipoprotein (HDL) is the major serum lipoprotein; hence, it is difficult to induce atherosclerosis in these species. We speculate that maintenance of the apoB mRNA editing activity in the adult liver may contribute to resistance to atherosclerosis in rodents and that the loss of this activity in senescent animals may predispose them to abnormal lipid metabolism.

We determined the extent of apoB mRNA editing by a differential hybridization method, using oligonucleotides and primer extension method after RT-PCR amplification of apoB mRNA. As we needed to precisely analyze a large number of samples, we did not use colony hybridization (17) or sequencing methods (30). Problems with accuracy occurred when samples containing extremely low or high percentages of apoB-100 mRNA were analyzed by differential hybridization or primer extension techniques (17), and we tested the accuracy of these two methods. Sufficient accuracy and linearity in the ranges below 10% and over 90% were obtained with the primer extension method. We decided to determine the extent of editing in the intestine (apoB-100 mRNA is usually below 10%) by the primer extension method.

In our preliminary experiments a considerable decrease in the extent of apoB mRNA editing, increases in apoB-100 protein, and expression level of apoB mRNA were observed after an overnight fast in 3-month-old SAM-R/1 and SAM-P/1 male mice. Percentage of hepatic apoB-100 mRNA, serum apoB-100 proteins, and hepatic apoB mRNA level of the mice fasted overnight were about 1.2, 1.4, and 3.0 times that of nonfasted control mice, respectively, in both strains. Intestinal apoB-100 mRNA

TABLE 1. ApoB mRNA editing in various tissues from SAM-P/1 mice

Tissues	Young (5 months)	Old (14 months)
	mean ● SD	
Kidney	47.85 ± 6.82[a]	63.80 ± 7.70
Stomach	19.32 ± 5.86	13.67 ± 6.63
Colon	29.85 ± 7.16	29.36 ± 3.72
Liver	30.43 ± 3.86[a]	41.32 ± 7.05

ApoB cDNA was amplified by RT-PCR from 5 μg of total RNA isolated from kidney, stomach, colon, and liver in the five young and five old male SAM-P/1 mice. The extent of apoB mRNA editing is expressed as a percentage of unedited apoB-100 mRNA to total apoB mRNA.

[a]Values for kidney and liver in young mice were significantly lower than those in old mice ($P < 0.05$, Student's t test).

and apoB mRNA levels remained unchanged with fasting. Since little is known of the effect of fasting on mice at the different stages of developmental and senescence, tissues and blood from nonfasted mice were isolated at a fixed time in the morning.

We used two inbred strains of mice, SAM-P/1 and SAM-R/1, developed in our laboratory for use in studies on aging. The SAM-P/1 strain with a short life span is a model of accelerated senescence. Age-associated senile amyloidosis (31, 32), chromosomal aberration (33), immunological deficiency (34), and increases in score of aging (20) appear in the SAM-P/1 strain in an accelerated manner compared with the findings in the SAM-R/1 strain, the latter thus being a good control strain with a normal aging process. Age-associated increases of unedited apoB-100 mRNA in the liver and apoB-100 protein in the serum from the SAM-R/1 strain were also observed in the SAM-P/1 strain, from a younger age and in an accelerated manner. The age-related changes observed here are thus universal and physiological aging phenomena in mice.

We also determined the extent of apoB mRNA editing activity in mice during the developmental stage in which lipogenesis changes rapidly and extensively. Exclusive accumulation of apoB-100 mRNA was noted in the liver during the prenatal period and a relatively rapid decrease of apoB-100 mRNA followed. On the other hand, the highest levels of expression of apoB mRNA were observed on the 7–10 postnatal days, both in the liver and intestine. The patterns of changes in abundance of mRNA did not coincide with the patterns of changes in the extent of apoB mRNA editing, either in the liver or intestine. In rats and humans, developmental regulation of hepatic and intestinal apoB-100 mRNA expression has been investigated (17, 35). The profiles of developmental changes (from fetal stage to 1-month-old) in apoB biogenesis, including RNA editing and mRNA level in mice, were almost the same as in rats. Furthermore, these profiles were similar in the SAM-R/1 and the SAM-P/1 strains (Figs. 3, 4, and 6). This would suggest that both SAM-P/1 and SAM-R/1 strains possess the same processes related to development. The apoB mRNA level was normalized relative to β-tubulin mRNA and relative β-tubulin mRNA levels decreased during development before 1 month of age. These changes in β-tubulin mRNA levels may not affect the validity of our findings, as the effect of the changes is the same on both SAM-R/1 and SAM-P/1 hepatic and intestinal apoB mRNA levels. Age-associated and accelerated decreases in serum levels of apolipoprotein A-II (apoA-II), a serum precursor for mouse senile amyloid fibrils, and HDL particles is a characteristic of lipoprotein biogenesis in the SAM-P/1 mice (36). On the other hand, significant age-related changes in total apoB levels were not observed in both strains in this and previous studies (36). The serum LDL cholesterol levels were almost constant during senescence in both strains. LDL cholesterol levels, determined by the heparin and manganese chloride method, in the 4- and 26-month-old SAM-R/1 mice and 4- and 14-month-old SAM-P/1 mice were 52.39 ± 12.04 mg/dl, 50.88 ± 7.40 mg/dl, 23.08 ± 5.67 mg/dl, and 27.60 ± 13.27 mg/dl, respectively (mean ± SD, n = 4) (36 and unpublished data, K. Higuchi, S. Ishikawa, K. Kitagawa, K. Kogishi, and T. Takeda). Reduction in the amount of HDL cholesterol in humans and mice fed a high cholesterol diet is a risk factor for atherosclerosis (37). The SAM-P/1 strain is therefore a good model for analysis of aging, especially the effects of aging on lipid biogenesis, since it has an accelerated senescence yet a normal developmental process.

The developmental and age-related changes in the proportion of apoB isoproteins followed changes in the extent of apoB mRNA editing in the liver. During senescence, changes in proportion of apoB-100 protein were more dramatic than changes in hepatic apoB-100 mRNA. As change in serum apoB distribution may reflect synthetic, secretional, and catabolic events, certain factors might accelerate changes caused by the change in hepatic apoB mRNA editing. Serum concentration of apoB-48 protein remained constant after the age of 2 months. As the mice were not fasted, apoB-48 protein derived from the intestine may contribute to maintain stable pools of apoB-48 protein.

Expression of apoB mRNA and editing activity in the human kidney, stomach, and colon was examined using the RT-PCR technique (35). ApoB mRNA editing activity was also revealed in several cell lines originating from nonhepatic and nonintestinal tissues (38). Although we did not detect apoB mRNA in tissues other than liver and small intestine using the slot-blot hybridization methods, and as also reported by Demmer et al. (7), the apoB DNA fragment was amplifiable from cDNA of mouse kidney, stomach, and colon and was not amplifiable from cDNA of other tissues. We used the SAM-P/1 strain to analyze the effects of aging on the extent of apoB mRNA editing in these tissues that expressed a small amount of apoB mRNA. There was a significant difference in the extent of editing in the kidney in old mice with lower editing activity.

A relatively large amount of apoB mRNA was detected in fetal membrane and placenta during the last stage of gestation. Large amounts of mRNA of apoB and other apolipoproteins were noted in rat and in human yolk sac (39). The finding that almost all of the apoB mRNA in mouse fetal membrane and placenta was unedited apoB-100 mRNA coincides with the result obtained in rats and humans (17, 35). Only apoB-100 protein was detected in amniotic fluid despite the presence of edited apoB-48 mRNA in the dam and in the fetal liver and intestine. ApoB in amniotic fluid might be synthesized by the placenta and fetal membrane and may not be derived

from the dam or fetal serum.

Whether or not the same mechanisms regulate developmental and age-associated modifications in the biogenesis of apoB remains unknown. More information is required on the nature of the mRNA editing itself before a satisfactory unifying hypothesis can be advanced concerning physiological regulation during development and senescence.

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